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# Detection of Multiple Polycyclic Aromatic Hydrocarbon–DNA Adducts by a High-Performance Liquid Chromatography–$^{32}$P-Postlabeling Method

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**A $^{32}$P-postlabeling procedure for identifying and quantifying hydrophobic DNA adducts was developed (by modifying the method of Randerath and co-workers) in which labeled adducts are separated by high-performance liquid chromatography (HPLC) and quantified by liquid scintillation counting. This method was first developed for fluoranthene–DNA adducts, and methods for optimal detection and quantification of DNA adducts with diol epoxide metabolites of benzo[*a*]pyrene (BPDE), chrysene (CHDE), and benz[*a*]anthracene (BADE) have now been established. Analytical conditions slightly different from those adopted for fluoranthene–DNA adducts are required for accurate quantification of BPDE–, CHDE–, and BADE–DNA adducts. In particular, HPLC analysis requires generation of nucleotide 5′-[$^{32}$P]monophosphate adducts by treatment with nuclease P1, and polycyclic aromatic hydrocarbon adducts demonstrate variable sensitivity to nuclease P1, mediated dephosphorylation. Thus, multiple adducts can be detected in one sample as long as the recovery of adducts under the applied conditions has been determined and chromatographic separation of labeled adducts is achieved. A battery of postlabeling assays can thus make it possible to detect optimally multiple adducts in one DNA sample. Results from these studies indicate that the HPLC–$^{32}$P-postlabeling assay is complementary to immunoassays in which related polycyclic aromatic hydrocarbon diol epoxide adducts cross-react for the quantification of adducts.**

## Introduction

Interspecies and interindividual variability in response to chemical carcinogens may be attributable in part to differences in the dose delivered to the target site. Many carcinogens bind covalently to DNA, and this DNA modification is thought to be a key initial event in mutagenesis and carcinogenesis. Thus, carcinogen-DNA adducts may be biologically important markers of the delivered dose.

Postlabeling assays are useful for quantifying DNA adducts formed *in vivo*. Recently, a version of the $^{32}$P-postlabeling procedure in which high-performance liquid chromatography (HPLC), instead of thin-layer chromatography, is used for separation of labeled adducts was described *(1)*. This method was used to detect and quantify fluoranthene (FA) adducts in the rat (*2*). FA, like other polycyclic aromatic hydrocarbons (PAH), is generally produced as a result of incomplete pyrolysis of organic material, resulting in widespread environmental exposure to complex mixtures of PAH. Therefore, application of the HPLC–$^{32}$P-postlabeling method in studies for monitoring human exposure to PAH requires chromatographic separation and quantitative recovery of multiple $^{32}$P-labeled PAH adducts in one sample. In recent studies, the HPLC $^{32}$P-postlabeling procedure has been extended for the detection of multiple PAH adducts. We chose to establish detection methods for benzo[*a*]pyrene (BaP), chrysene (CH) and benz(*a*)anthracene (BA) diol epoxide (DE) adducts, because these adducts, as well as others, cross-react in a widely applied immunoassay *(3–5)*. Thus, the postlabeling method described herein would be complementary to that immunosssay.

## Methods and Results

### The HPLC–$^{32}$P-Postlabeling Procedure for FA Adducts

The HPLC–$^{32}$P-postlabeling method first developed for quantative analysis of FA adducts *(1)* is outlined in Figure 1. Briefly, carcinogen-modified DNA is hydrolyzed enzymatically with micrococcal nuclease and spleen phosphodieterase to nucleotide 3′-monophosphate adducts and nucleotide 3′-monophosphates. Adducts are separated from unmodified nucleotides by application of the hydrolysate to a disposable C18 cartridge. More than 99% of the unmodified nucleotides are removed in an aqueous wash, and adducts are eluted with methanol (data not shown).

Complete removal of unmodified nucleotides is essential for reproducible quantification of FADE adducts by postlabeling.

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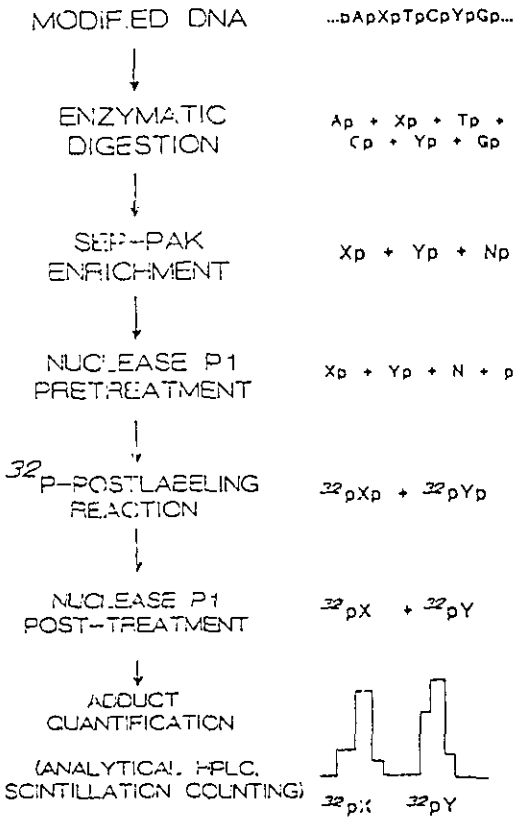


FIGURE 1. The high-performance liquid chromatography (HPLC)-$^{32}$P-postlabeling procedure for detection of radiolabeled nucleotide 5′-monophosphate adducts. X and Y represent modified nucleosides. Modified from Gorelick and Wogan *(1)*.

Thus, to eliminate the residual nucleotides as substrates in the labeling reaction, the 3′-phosphate moiety is selectively removed from unmodified nucleotides by a brief (15–30 min) digestion with nuclease P1 immediately prior to postlabeling. This step only minimally affects recovery of FADE adducts *(1)*.

Transfer of the $^{32}$P-label from [$\gamma$-$^{32}$P]ATP to the 5′-hydroxyl position of adducts is mediated by T4 polynucleotide kinase, producing 3′-[5′-$^{32}$P]bisphosphates. Reverse-phase HPLC is employed to exploit the hydrophobicity of FAH adducts and thus to separate the multiple adducts that may be found in one sample. Conversion to nucleotide [5′-$^{32}$P]monophosophates, which improves reverse- phase HPLC separation of adducts, is accomplished by prolonged treatment, with nuclease P1, to which FADE adducts are sensitive. Finally, adducts are separated by reverse-phase HPLC, and adduct levels are determined by liquid scintillation counting.

Each step in this procedure was optimized previousiy for the detection of FADE adducts *(1)*; the most important requirement to maximize recovery of labeled adducts was complete separation of normal and modified nucleotides prior to postlabeling. Most of the steps in which FA adduct loss occurs have been identified *(1)*. Overall recovery is expected to be 15–35% of the DNA-bound FA at modification levels of one adduct in $10^6$-$10^7$ nucleotides. Since 10–15% of the initial DNA-bound FA is actually recovered, loss of at most 25% of the DNA-bound FA is unassigned.

## HPLC-$^{32}$P-Postlabeling Assay for Detection of BPDE, BADE, and CHDE Adducts

Attempts to analyze DNA modified *in vitro* with diol epoxides of BP, BA, or CH (Fig. 2) by the HPLC-$^{32}$P- postlabeling method exactly as previously described *(1)* i.e., with nuclease P1 pretreatment for 15 min and nuclease P1 post-treatment for 16–18 hr, were unsuccessful; $^{32}$P-labeled adducts were not detected (data not shown). Because the cornerstone of this method is the balance between adduct resistance to brief nuclease P1-mediated dephosphorylation and adduct suceptibility to extensive nuclease P1-mediated dephosphorylation, the sensitivity of each of these PAH diol epoxide adducts to nuclease P1-mediated dephosphorylation was investigated.

Chromatographic profiles of a $^{32}$P-labeled adducts and the appropriate ultraviolet markers are shown in Figure 3. In each case, a major $^{32}$P peak co-eluted with the appropriate authentic nucleotide 5′-monophosphate adduct. No qualitative difference was observed in any of the chromatographic profiles associated with either nuclease P1 pretreatment or the duration of nuclease P1 post-treatment (data not shown).

The effect of nuclease P1 pretreatment on recovery of $^{32}$P-labeled adducts was determined. The improvement in recovery of BPDE, BADE, and CHDE adducts by inclusion of a 10-min nuclease P1 pretreatment step is indicated recovery improved by at least 6-fold as much as 20-fold (BPDE). Analyses for $^{32}$P-

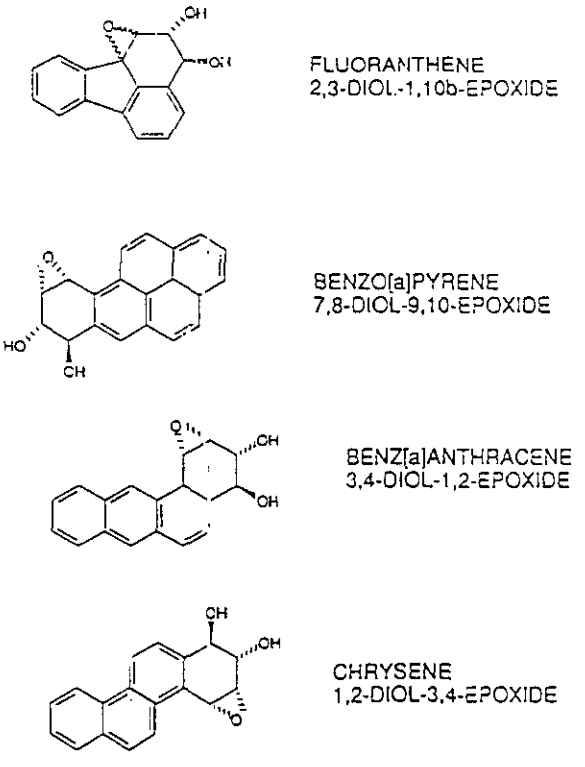


FIGURE 2. Structures of the polycyclic aromatic hydrocarbon diol epoxide metabolites reacted with DNA. The major adduct for each metabolite is an $N^2$-guanine adduct. Relative stereochemistry is shown.

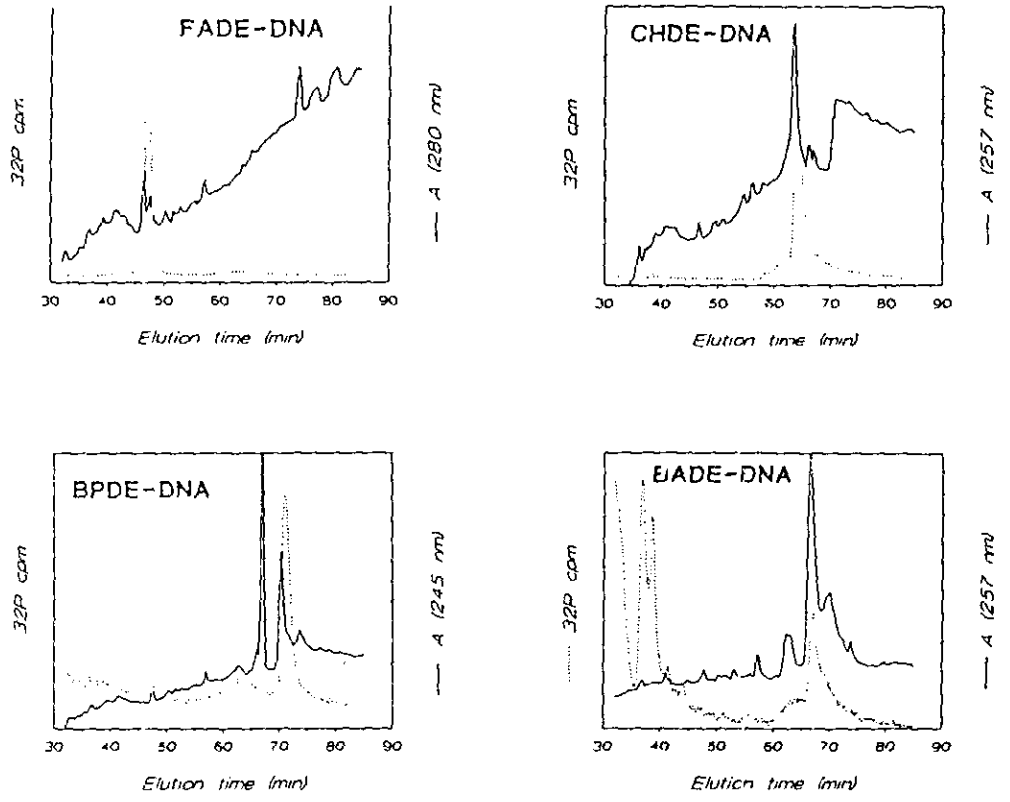


FIGURE 3. Chromatographic profiles of $^{32}$P-labeled adducts from DNA modified with fluoranthene (FADE), benzo[*a*]pyrene (BPDE), chrysene (CHDE), and benz(*a*)anthracene (BADE) diol epoxides *in vitro*. Adducts were postlabeled with a 10-min nuclease P1 pretreatment and 2.5-hr nuclease P1 post-treatment. One-third of each sample (18.4 μL) was mixed with an appropriate amount of the corresponding unlabeled nucleotide 5'-monophosphate adduct (for ultraviolet marker) and injected via a WISP Model 715 autoinjector (Waters Associates). Chromatographic separation on a μBondapak C18 column [3.9 mm (ID) x 30 cm, Waters Associates] at room temperature was facilitated by the following gradient (1 mL/min). Isocratic elution with solvent A for 20 min preceded a linear gradient over 10 min to 20% solvent B and a linear gradient from 20% to 60% B over 45 min. Flow was held at 60% B for 10 min prior to 25-min equilibration in the initial solvent. Solvent A: 10% methanol in 0.1 M ammonium acetate, 1 mM ammonium dihydrogen phosphate, pH 5.7; solvent B: methanol. Radioactivity was detected on-line with either a Model A140 or Model A280 detector (Radiomatic Instruments and Chemical Co, Inc., Meriden, CT) equipped with a 0.5-mL liquid flow cell, at 3 mL/min Flo-Scint IV.

labeled unmodified nucleotides showed that a 10-min nuclease P1 pretreatment step is indicated in Table 1. Adduct recovery improved by at least 6-fold (BADE) and as much as 20-fold (BPDE). Analyses for $^{32}$P-labeled unmodified nucleotides showed that a 10-min nuclease P1 pretreatment is sufficient to dephosphorylate residual unmodified nucleotides (data not shown).

Figure 4 shows the dependence of $^{32}$P-labeled adduct recovery on the length of nuclease P1 post-treatment. Although prolonged hydrolysis with nuclease P1 is required for maximal recovery of FADE adducts (12–18 hr) *(1)*, maximal recovery of BPDE and CHDE adducts occurs after less than 30-min nuclease P1 post-treatment, and maximal recovery of BADE adducts occurs after 1–2 hr post-treatment. Thus, BPDE, CHDE, and BADE adducts are less resistant to nuclease P1-mediated dephosphorylation than FADE adducts.

**Table 1. Effect of nuclease P1 pretreatment on recovery of $^{32}$P-labeled adducts.[a]**

| Pretreatment[b] | $^{32}$P-labeled adduct, fmole[c] | | |
|---|---|---|---|
| | BPDE | CHDE | BADE |
| No nuclease P1 | 49± 9 | 49± 5 | ND |
| Nuclease P1 | 313 ±70 | 468±89 | 43± 4 |

Abbreviations: BPDE, benzo[*a*]pyrene diol epoxide; CH, chrysene; BADE, benz[*a*]anthracene diol epoxide; ND, none detected (limit of detection was approximately 0.4 fmole adduct).

[a]All samples were also treated with nuclease P1 for 30 min after labeling. Average ± SD for three to four samples.

[b]For 10 min with 12.5 μg nuclease P1 or water.

To determine the recovery of $^{32}$P-labeled BPDE adducts in the HPLC-$^{32}$P-postlabeling procedure, increasing amounts of [$^3$H]BP-modified DNA were mixed with carrier DNA (calf thymus) to comprise samples of 15 μg DNA. Recovery of $^{32}$P-labeled BaPDE adduct was linear for DNA samples containing 20-660 fmole BaP adduct [$r$ = 0.876 (Fig. 5)]. Recovered $^{32}$P-labeled BPDE adducts constituted about 10% of the original DNA-bound BaP; thus, approximately 12% of the BPDE adducts originally in the DNA were recovered by postlabeling. Losses of BPDE adducts have not been assigned to particular steps in the postlabeling procedure; however, nonspecific losses due to sample manipulation are likely, as in the case of FADE adducts. Adduct recovery is currently being improved.

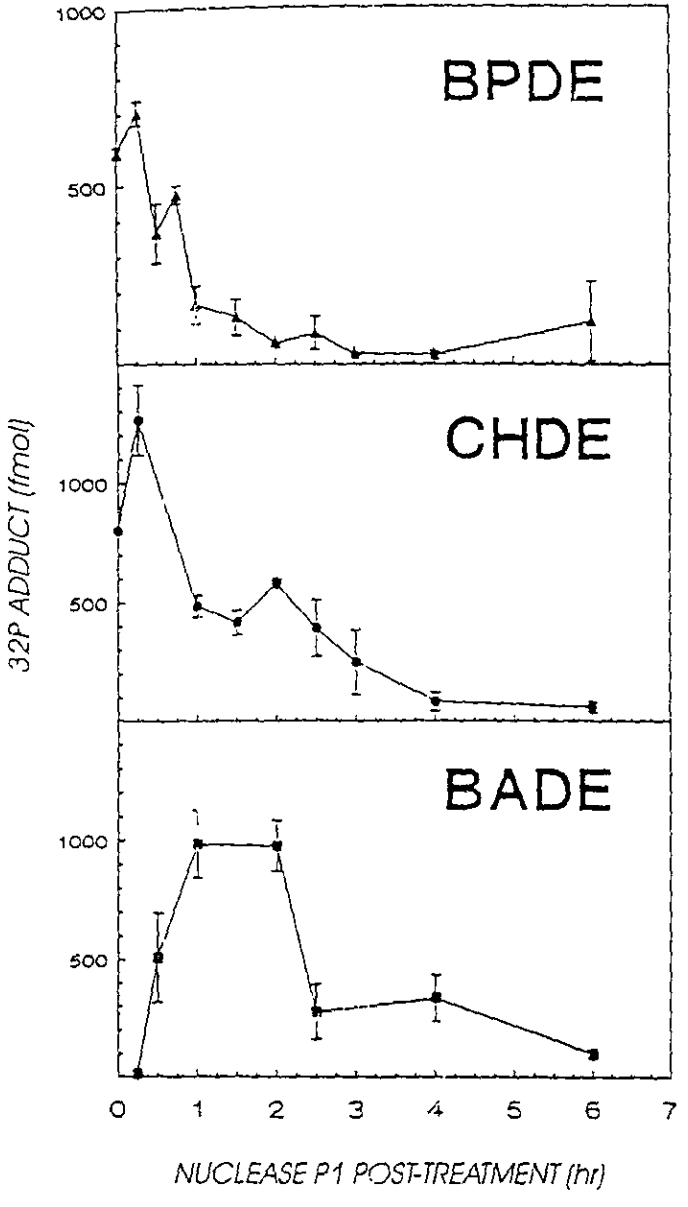


FIGURE 4. Time course for nuclease P1 treatment after $^{32}$P-labeling of benzo[*a*]pyrene diol epoxide (BPDE)-DNA, chrysene diol epoxide (CHDE)-DNA, and benz[*a*]anthracene diol epoxide (BADE)-DNA. Each diol epoxide-modified DNA (1 μg) was mixed with calf thymus DNA (14 μg) and postlabeled with a 10-min nuclease P1 pretreatment as described *(1)*. The mean and range for duplicate samples are indicated.

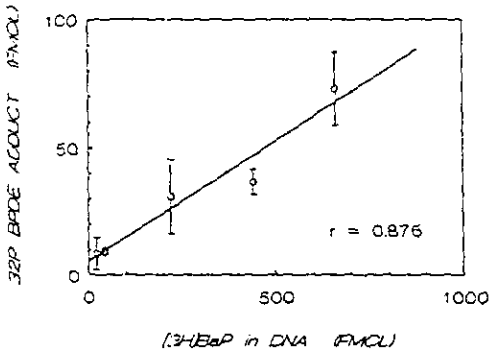


FIGURE 5. Effect of the amount of [$^3$H]benzo[*a*]pyrene (BaP)/DNA on recovery of $^{32}$P-labeled BaP-diol epoxide (BPDE) adducts. DNA was modified *in vitro* with [$^3$H]BaP as described by Dankovic et al. *(6)*, whereby the major adduct co-eluted with the (+) *anti*-diol epoxide adduct of deoxyguanosine and accounted for approximately 80% of the total bound metabolites. Increasing amounts of [$^3$H]BaP-modified DNA were mixed with calf thymus DNA, and 15 μg DNA samples were postlabeled with a 10-min nuclease P1 pretreatment and a 30-min nuclease P1 post-treatment. Duplicate or triplicate samples were analyzed twice. Average and range are presented. The equation of the regression line is $y = 0.094x + 5.4$.

## Discussion

Many versions of the $^{32}$P-postlabeling assay have been developed to detect adducts of known and unknown structure; most of these involve thin-layer chromatography for adduct separation *(7,8)*. The time-consuming, labor-intensive nature of thin-layer chromatography, and the fact that quantitative estimates of adduct recovery after this procedure have not been determined rigorously, limits its application in molecular epidemiology studies.

Coupling postlabeling with HPLC offers technical advantages for adduct detection and quantification. The advantages for adduct separation include speed of analysis, reproducible and characteristic retention, quantification of low adduct levels by on-line analysis of radioactivity for minimal human exposure to $^{32}$P, the ease with which a sample can be recovered after separation, and the possibility of coupling HPLC separation with various other detection methods, such as mass spectrometry, which can provide additional structural information. Reverse-phase HPLC has been used for analyzing postlabeled aromatic *(9;* this work) as well as alkyl *(10,11)* nucleotide monphosphate adducts. Alternatively, ion-pair HPLC can be used for analyzing postlabeled bulky nucleotide bisphosphate adducts *(12,13)*.

The most sensitive versions of the postlabeling assay include a step to separate modified and unmodified nucleotides prior to labeling; alternative versions of the postlabeling method involving different techniques. Nuclease P1 treatment of hydrolyzed DNA (without intial chromatographic separation) before labeling is applicable for selected adducts *(14)*, whereas butanol extraction is an efficient method for isolating other selected adducts *(15,16)*. Adduct enrichment by HPLC fractionation is advantageous in that the yield of nucleotides actually in the sample can be measured and the relative adduct level in the sample can be calculated *(17-20)*. Immunoaffinity chromatography may be useful for isolating adducts, although its application is limited by solvent incompatibilities *(21)* and antibody availability. The results described above show the value of combining of methods for complete elimination of unmodified nucleotides.

BPDE, BADE, and CHDE adducts are significantly more sensitive to dephosphorylation by nuclease P1 than are FADE adducts. Similar differences in sensitivity to nuclease P1 has been observed with other classes of adducts, e.g., arylamine-C$^8$-guanine adducts *(16)*. It is notable that adducts of the same chemical and nucleotide class, i.e., PAH diol epoxide-N$^2$-guanine adducts, vary widely in their sensitivity to nuclease P1. These results underscore the fact that the balance between adduct resistance to brief nuclease P1 pretreatment and prolonged

nuclease P1 post-treatment must be examined for each adduct. A battery of postlabeling assays may make it possible to detect optimally multiple adducts in one DNA sample. Alternatively, determination of the recovery of multiple adducts under particular conditions will enable quantitative analysis, albeit sometimes with less than optimal detection.

Application of the HPLC-$^{32}$P-postlabeling method to analysis of adducts in human tissues requires the ability to detect and quantify simultaneously multiple adducts, often from the same chemical class. Thus, we undertook to optimize this sensitive method for the detection of multiple PAH adducts as well as to determine the quantitative recovery of each adduct in this procedure. Our results indicate that documentation of the quantitative power of this assay is essential for accurate interpretation of adduct levels determined in human samples.

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